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Inhibition of Carboxyethylphosphoramide Mustard Formation from 4-Hydroxycyclophosphamide by Carmustine

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ABSTRACT It has been reported that the toxicity of carmustine (BCNU)/cyclophosphamide (CY)/etoposide regimen (when BCNU is split into 4 doses) is less than that of BCNU/CY/cisplatin regimen (when the same amount of BCNU is administered as a single dose). We hypothesized that this might in part be due to the inhibition of aldehyde dehydrogenase 1 (ALDH1) by BCNU or its degradation product, 2-chloroethyl isocyanate, which is likely to be more pronounced at the higher BCNU dose. The effects of BCNU and 2-chloroethyl isocyanate on the formation of carboxyethylphosphoramide mustard (CEPM) from 4-hydroxycyclophosphamide (HCY) was evaluated in human liver cytosol incubations. We found that CEPM formation from HCY was inhibited strongly by BCNU and weakly by 2-chloroethyl isocyanate. The mechanism of inhibition of ALDH1 activity by BCNU was elucidated using indole-3-acetaldehyde (IAL) as the probe substrate in ALDH1 prepared from human erythrocytes. BCNU was a competitive inhibitor of ALDH1 activity with a K_i of 1.95 μM. The inhibition was independent of preincubation time and reversible by dialysis. The calculated %inhibition of ALDH1 activity by acrolein and BCNU in patients receiving BCNU in 4 split doses with CY was 81%, and it increased to 92% in single dose BCNU regimen. Thus, the calculation indicates that residual operating ALDH1 activity is halved in the presence of single-dose BCNU compared to split-dose BCNU. The inhibition of ALDH1 may contribute to the observed lower incidence of toxicity when BCNU was split into 4 doses compared with single dose and coadministered with CY although dose-dependent effects of BCNU on glutathione and glutathione reductase are also likely to contribute.

Key Words: cyclophosphamide carmustine ALDH1 4-hydroxycyclophosphamide alkylator

Abbreviations: CY, cyclophosphamide, BCNU, carmustine, HCY, 4-hydroxycyclophosphamide, CEPM, carboxyethylphosphoramide mustard, ALDH1, aldehyde dehydrogenase 1.

INTRODUCTION

Cyclophosphamide (CY) is one of the most frequently used alkylating agents in the treatment of malignancy and in preparative regimens for bone marrow transplantation. It is a prodrug that is oxidized in human liver by CYP2C and CYP3A to form 4-hydroxycyclophosphamide (HCY) or by unidentified P450 isoform(s) to form deschloroethylCY (DCCY) and chloroacetaldehyde (1,2). HCY is the major active circulating metabolite, which enters cells and decomposes (through its tautomer aldophosphamide) to phosphoramide mustard (PM) and acrolein. PM is a bifunctional alkylator of DNA, the ultimate cytotoxic metabolite of cyclophosphamide. Alternatively, HCY is detoxified to (in decreasing order of importance) carboxyethylphosphoramide mustard (CEPM) by aldehyde dehydrogenase 1 (ALDH1) (3), alcophosphamide by aldose reductase (4) and 4-ketocyclophosphamide by cytochrome P450 (5) (Figure 1). In the hematopoietic stem cell transplantation setting, CY administration is associated with cardiac, gastrointestinal, pulmonary, urinary tract, and hepatic toxicities (5).

Carmustine (BCNU) is an antineoplastic drug widely used for the treatment of solid tumors and lymphomas and in preparative regimens for bone marrow transplantation (6). BCNU decomposes chemically under physiological conditions to yield 2-chloroethyl isocyanate and the chloroethyl carbonium ion intermediate, which is believed to be the alkylating moiety (7,8) (Figure 1). The major toxicities of BCNU in the hematopoietic stem cell transplantation setting are to the lung, liver and kidney (7).

The schedule of BCNU administration in combination with two other chemotherapeutic agents (one is CY, another one is cisplatin or etoposide) has been found to be a significant risk

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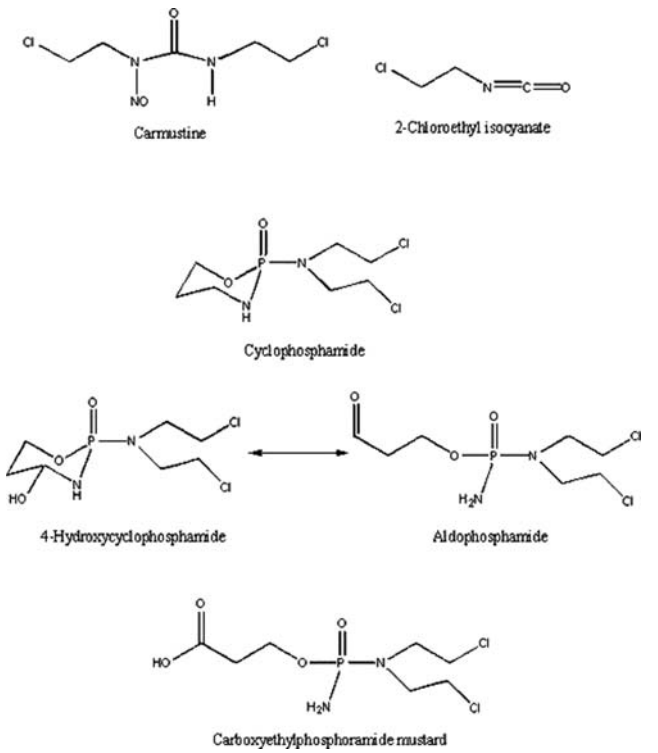


Figure 1. Structures of the drugs and metabolites referred to in the text.

factor for the development of venoocclusive disease of the liver (VOD) (9). Seven out of 42 patients receiving BCNU as a single dose (CY was given by 1-hr infusion once a day for 4 days and BCNU was given by 2-hr infusion on the day that the last CY dose was given, the total doses of CY and BCNU were 5,625 - 7,500 and 450 - 600 mg/m^2) developed VOD. One out of 53 patients receiving the same amount of BCNU in split doses over 4 days (CY was given by 1-hr infusion twice a day for 4 days and BCNU was given by 2-hr infusion once a day on the same days that CY was given, the total doses of CY and BCNU were 4,500 - 7,200 and 450 - 600 mg/m^2) developed VOD. Neither BCNU nor CY themselves were observed to have a significant effect on the incidence of VOD by multivariable logistic regression analysis. The authors suggested that there might be interactions between BCNU and other alkylating agents when used in combination, as VOD is seen more frequently with BCNU doses of 450 - 600 mg/m^2 in combination with CY but less frequently even at doses greater than 1200 mg/m^2 when given alone.

Some alkyl isocyanates have been shown to be inhibitors of ALDH in rats . Thus, 2-chloroethyl isocyanate, a decomposition product of BCNU, might inhibit this critical enzyme thereby increasing the exposure to HCY at a given dose of CY. The objectives of this investigation were to determine whether the formation of CEPM from HCY was inhibited by BCNU and its decomposition product 2-chloroethyl isocyanate, and to elucidate the mechanism of inhibition.

MATERIALS AND METHODS

Materials

Indole-3-acetaldehyde (IAL), indole-3-acetic acid (IAA) and NAD were purchased from Sigma Chemical Company (St. Louis, MO). BCNU was purchased from Bristol Laboratories (Princeton, NJ). Acrolein and 2-chloroethyl isocyanate were purchased from Aldrich Chemical (Milwaukee, WI). Carboxyethylphosphoramide mustard (CEPM) was a generous gift from ASTA Medica AG (Frankfurt, Germany). 4-Hydroperoxycyclophosphamide and tetradeuteurocarboxyethylphosphoramide mustard (d_4-CEPM) were prepared in our laboratory by published methods (11).

Preparation of Human Liver Cytosol. Human livers were obtained from the human liver bank in the Departments of Pharmaceutics and Medicinal Chemistry at the University of Washington (Seattle, WA). Liver was homogenized in 100 mM potassium phosphate buffer (pH 7.4) and centrifuged at 10,000 x g, 4° C for 30 min. The supernatant was filtered through 6-ply surgical gauze and centrifuged at 100,000 x g, 4° C for 60 min. The resulting supernatant cytosolic fraction was stored at -70° C until use. Protein concentration was determined with Bio-Rad (Oakland, CA) protein assay reagent, with bovine serum albumin as the standard (12).

Preparation of ALDH1 from Erythrocytes. Human erythrocytes were obtained from Puget Sound Blood Center and Program (Seattle, WA). Erythrocytes were hemolyzed and ALDH1 prepared with a DEAE-cellulose column as described previously (13).

Incubations

The effect of BCNU and 2-chloroethyl isocyanate on the formation of CEPM from HCY was investigated in human liver cytosolic incubations. 4-Hydroperoxycyclophosphamide (5 mM) was

reduced to HCY with the addition of sodium thiosulfate (25 mM) and allowing the mixture to stand on ice for 1 hr immediately before incubation. CEPM formation was evaluated in an incubation system containing 20 μM HCY (approximately its K_m for ALDH1), 1 mg/ml cytosolic protein, 0.5 mM NAD and BCNU (30 μM, approximately its plasma C_{max} in patients receiving 600 mg/m^2 BCNU) or 2-chloroethyl isocyanate (30 μM) in 100 mM potassium phosphate buffer (pH 7.4) (total volume 0.5 ml). Control incubations contained no inhibitors. All incubations were performed in duplicate. The reaction mixture was preincubated at 37° C for 2 min before the reaction was started by the addition of NAD. The incubation was stopped after 5 min. CEPM was measured by LC/MS (14).

The formation rate of IAA from IAL was used to measure ALDH activity. Duplicate incubations were performed at 37° C in 100 mM potassium phosphate buffer (pH 7.4) containing IAL (2 μM, approximately its K_m for ALDH1), BCNU (2 - 32 μM, selected based on plasma concentrations in patients receiving BCNU) or 2-chloroethyl isocyanate (2 - 32 μM) and human liver cytosol (0.02 mg cytosolic protein/ml) in a total volume of 0.5 ml. Control incubations contained no inhibitors. After preincubation for 2 min, reactions were initiated by the addition of 0.5 mM NAD and stopped after 5 min by the addition of 50 μ l of 1 N sodium hydroxide and 100 μ l of 10% zinc sulfate. In preincubation time-dependent experiments, human liver cytosol and BCNU were preincubated at 37° C for 10 min, then the reaction was initiated with the addition of IAL and NAD, and stopped after 5 min. The sample was sealed, vortexed, left on ice for 5 min and centrifuged for 5 min. Twenty μ l of the supernatant was injected to a Hewlett-Packard 1050 series HPLC equipped with a Rainin Microsorb C18 column and a fluorescence detector (excitation: 278 nm, emission: 359 nm) to measure IAA. The mobile phase was 27% acetonitrile and 73% of 25 mM ammonium acetate buffer (pH 4.0), delivered at a rate of 0.9 ml/min. The retention time of IAA was 3.7 min and the run time was 5 min. The concentration of IAA was quantified by peak height.

The mechanism of ALDH1 inhibition by BCNU was investigated in ALDH1 prepared from human erythrocytes. Duplicate incubations were performed at 37° C in 100 mM potassium phosphate buffer (pH 7.4) containing IAL (0.25 - 1.5 μM), BCNU (0 - 12 μM), and erythrocyte ALDH1 in a total volume of 0.5 ml. After preincubation for 2 min, reactions were initiated by the addition of 0.5 mM NAD and stopped after 5 min.

Dialysis

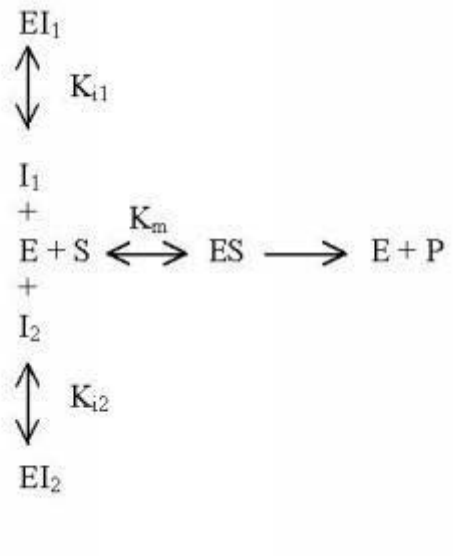
Dialysis experiments were performed to examine the reversibility of the inhibition of ALDH1 activity by BCNU. ALDH1 prepared from human erythrocytes was preincubated in the absence or presence of BCNU (12 μM) for 5 min at 37° C. At the end of the preincubation, samples were taken and split for the immediate measurement of IAA formation rate, while the remainder was dialyzed for 6 hr at 4° C against 1 liter of 100 mM potassium phosphate buffer (pH 7.4) using Sprectra/Por Molecularporous® membrane tubing, molecular weight cutoff 3500 D (The Spectrum Companies, Gardena, CA). The dialysis buffer was changed at 2 hr. IAA formation rate was measured at the end of dialysis.

Data Analysis

The inhibition of IAA formation by BCNU was first analyzed by Lineweaver-Burk plot. A competitive inhibition model (15) thus was found to be appropriate to fit the data:

The value of K_i was estimated by fitting this model to the untransformed IAA formation rate and IAL concentration data using the WinNonlin program (Scientific Consulting, Inc., Cary, NC). A weight of V^{-1} was used in the iterative fitting process.

We have found previously that acrolein was a competitive inhibitor of ALDH1 (16). Since acrolein is a metabolite of CY, ALDH1 is likely to be inhibited after the administration of CY if acrolein concentration is significant relative to its K_i. Thus, the %inhibition of ALDH1 activity in vivo in patients receiving CY and BCNU by the different schedules of administration was calculated with in vivo substrate and inhibitor concentrations and in vitro constants (determined with IAL as the substrate) according to an inhibition model (below). The inhibition model treats acrolein and BCNU as mutually independent competitive inhibitors of ALDH1:



$$\%Inhibition = \left[1 - \frac{K_m + S}{K_m * \left(1 + \frac{I_1}{K_{i1}} + \frac{I_2}{K_{i2}}\right) + S}\right] * 100\%$$

where K_m is the Michaelis constant for HCY for the enzyme, S is the concentration of HCY, K_{i1} and K_{i2} are the inhibition constants for BCNU and acrolein, and I_1 and I_2 are the concentrations of BCNU and acrolein, respectively.

RESULTS

The concentration-dependent inhibition of ALDH activity (as measured by IAA formation) by BCNU and its decomposition product 2-chloroethyl isocyanate is shown in Figure 2. ALDH activity determined with 2 µM IAL (approximately its K_m for ALDH1) as substrate was inhibited by 71% with 32 µM BCNU (approximately its plasma C_{max} at a dose of 600 mg/m^2) and 20% with 32 µM 2-chloroethyl isocyanate. Preincubation of BCNU with human liver cytosol for 10 min did not increase the degree of inhibition compared with no preincubation, suggesting that decomposition to 2-chloroethyl isocyanate was not the cause of inhibition observed with the addition of BCNU.

The effects of BCNU and 2-chloroethyl isocyanate on the formation of CEPM from HCY in human liver cytosol incubations are shown in Table 1. BCNU at 30 µM inhibited CEPM formation rate from HCY (at 20 µM, approximately its K_m for ALDH1) by 74 ± 2%, while 2-chloroethyl isocyanate (30 µM) inhibited by only 11 ± 11% (n = 3).

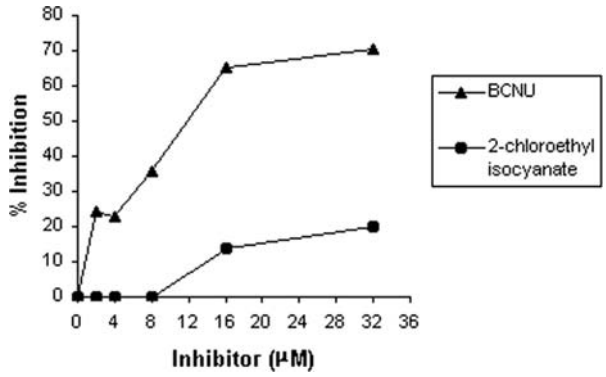


Figure 2. Concentration-dependent inhibition of ALDH activity by BCNU and 2-chloroethyl isocyanate. Human liver cytosol (0.02 mg protein/ml), IAL (2 µM) BCNU (0-32µ) or 2-chloroethyl isocyanate (0-32µ) were preincubated at 37°C for 2 minutes, and the reaction was initiated with the addition of NAD (0.5 mM) and stopped after 5 minutes. IAA formation rate in the control (without inhibitor) was 15 nmol/min/mg protein. Data are means of duplicate determinations.

Table 1. Effects of BCNU and 2-chloroethyl isocyanate on the formation of CEPM from HCY in human liver cytosol incubations

Human liver cytosol	**% inhibition**	
	BCNU	**2-chloroethyl isocyanate**
	30 µM	**30 µM**
127	74	12
133	76	21
144	73	0
mean	74	11
SD	2	11

Human liver cytosol, HCY (20 µM) and BCNU (30 µM) or 2-chloroethyl isocyananate (30 µM) were preincubated at 37°C for 2 minutes, then the reaction was initiated with the addition of NAD (0.5 mM) and stopped after 5 minutes. Control incubations contained no inhibitors. The mean control activity was 0.61 nmol/minute/mg protein in the three livers. Values reported for individual livers are mean of duplicate determinations.

Since BCNU was a more potent inhibitor of ALDH activity than 2-chloroethyl isocyanate with respect to both IAA and CEPM formation, it was further investigated to determine its mechanism of inhibition of ALDH1, the major ALDH isoform responsible for the formation of CEPM from HCY.

The mechanism of ALDH1 inhibition by BCNU was investigated in ALDH1 prepared from human erythrocytes. The Lineweaver-Burk plot and the replot of the slope indicated a competitive inhibition mechanism (Figure 3). A competitive inhibition model thus was used to fit the untransformed IAA formation rate and IAL concentration data. The estimated value of K_i was 1.95 μM. The model-predicted vs observed IAA formation rate as a function of IAL concentration at various concentrations of BCNU is shown in Figure 4. The reversibility of the inhibition by BCNU was evaluated by dialysis. The IAA formation rate (at 1.5 μM IAL) in incubations with 12 μM of BCNU was 25.7% of the control activity before dialysis, and it increased to 99.5% of the control activity after 6 hr of dialysis at 4° C.

Table 2. Calculated percentage inhibition of ALDH1 activity in vivo in patients receiving CY and BCNU

	Split-dose BCNU	**Single-dose BCNU**
Peak plasma concentration (μM)		
HCY	2.1	2.1
Acrolein	1.6	1.6
BCNU	5.1	20.4
% Inhibition due to		
Acrolein	67	67
BCNU	69	90
Acrolein + BCNU	81	92

Percentage inhibition was calculated with in vivo substrate and inhibitor concentrations and in vitro inhibition constants (obtained using IAL as a substrate) according to the model shown in Methods. *Ki* was 1.95 μM for BCNU and 0.646 μM for acrolein; *Km* was 12.9 μM for HCY. *S* for HCY and I for acrolein and BCNU were the values as shown in the table.

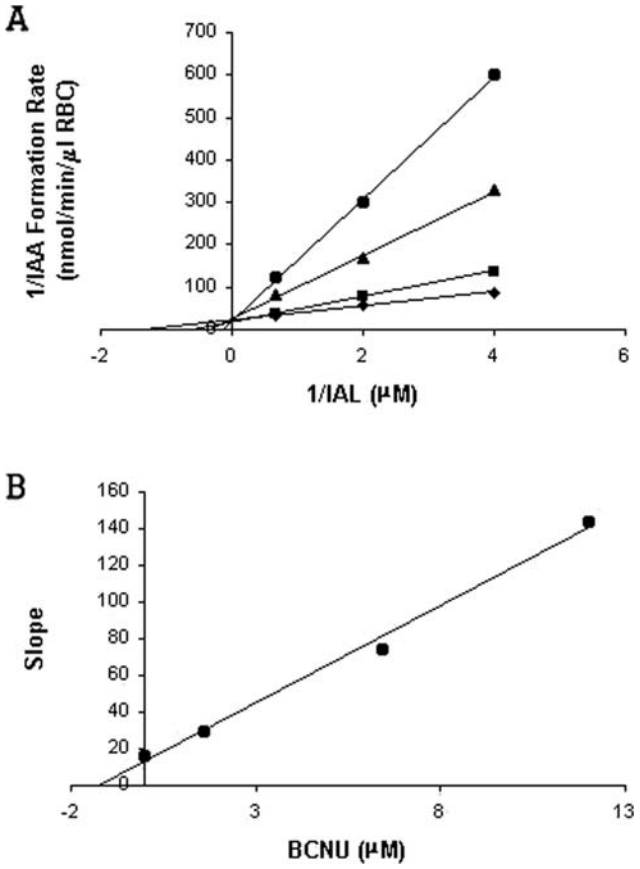


Figure 3. *Upper panel:* Lineweaver-Burk plot for inhibition of IAA formation by BCNU in ALDH1 prepared from human erythrocytes. *Lower panel:* Replot of the slope of Lineweaver-Burk plot at various concentrations of BCNU. Data are means of duplicate determinations.

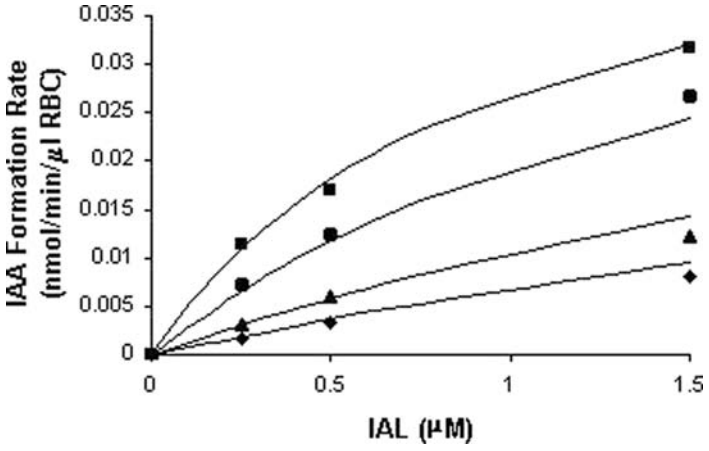


Figure 4. Model predicted (line) versus observed (symbol) IAA formation rate as a function of IAL concentration at various concentrations of BCNU in ALDH1 prepared from human erythrocytes.

The calculated %inhibition of ALDH1 activity in vivo in patients receiving CY and BCNU is shown in Table 2. In the split-dose BCNU regimen, BCNU was calculated to cause 69% inhibition of ALDH1 activity if acrolein were not present. In the absence of BCNU, ALDH1 activity was inhibited by 67% due to acrolein. In the presence of both acrolein and BCNU, ALDH1 activity was inhibited by 81%. In the single-dose BCNU regimen, BCNU was calculated to cause 90% inhibition of ALDH1 activity if acrolein were not present. In the presence of both acrolein and BCNU, ALDH1 activity was inhibited by 92%. The %remaining ALDH1 activity thus was 19% and 8% in the regimens with split-dose and single-dose BCNU, respectively. The remaining ALDH1 activity in single-dose BCNU regimen was calculated to be less than half of that in split-dose regimen.

DISCUSSION

The major finding of this investigation was that CEPM formation from HCY was strongly inhibited by BCNU and weakly by 2-chloroethyl isocyanate at therapeutically relevant concentrations in human liver cytosol incubations. BCNU was a reversible competitive inhibitor of ALDH1 prepared from human erythrocytes. These results and previous findings that acrolein, a product formed from HCY, is also a competitive inhibitor of ALDH1 (16), allowed a calculation of the extent to which this enzyme would be inhibited under two clinical BCNU dosing schemes. The residual activity of ALDH1 when BCNU was included in the model at concentrations associated with the administration of a single dose (450 - 600 mg/m^2) of the drug was half the activity when the same total dose of BCNU was divided into 4 equal doses administered over 4 days.

Acrolein derived from HCY has been suggested to account for toxicity to sinusoidal endothelial cells and at least partially to account for VOD in cytotoxic regimens containing CY (17). The finding that BCNU inhibits ALDH1 suggests one mechanism by which BCNU might increase the toxicity of CY. However, the inhibition of ALDH1 is not the only means by which the toxicity of CY might be potentiated by BCNU.

BCNU itself is a potent inactivator of glutathione reductase, an enzyme that reduces glutathione disulfide to glutathione, thereby contributing importantly to the homeostasis of this critical nucleophile. Glutathione is an important scavenger of electrophiles such as acrolein (18) and has been shown to be a critical factor in the toxicity of CY to hepatocytes (17). The BCNU electrophilic toxic decomposition product 2-chloroethyl isocyanate is also scavenged by glutathione by forming a glutathione conjugate. Of particular importance to the consideration of the role of BCNU dose in the interaction between BCNU and CY is the observation made by several investigators that BCNU inactivates glutathione reductase at relatively low concentrations in human erythrocytes and K562 leukemia cell line in vitro, while substantially higher concentrations (approximately 10 fold) are required to actually deplete intracellular GSH concentration (19,20). Thus, there are at least 3 mechanisms by which BCNU can potentiate the toxicity of CY: (1) inhibition of ALDH1, (2) inactivation of glutathione reductase and (3) consumption of glutathione through conjugation with 2-chloroethyl isocyanate. Each of these mechanisms appears to be dose-dependent.

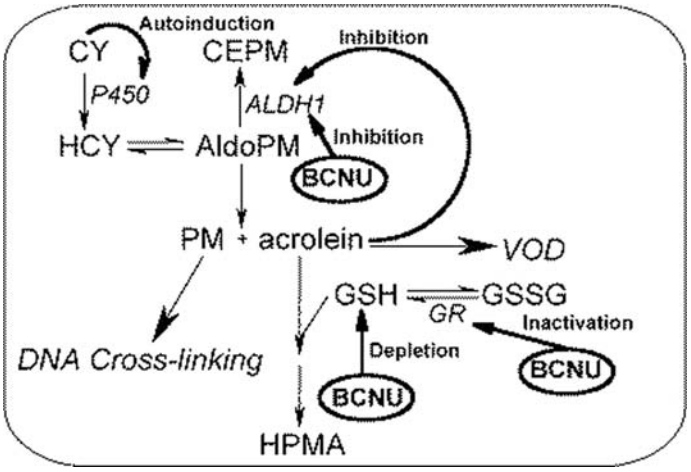


Figure 5. A scheme of CY/BCNU regimen and VOD. CY, cyclophosphamide; HCY, 4-hydroxycyclophosphamide; AldoPM, aldophosphamide; CEPM, carboxyethylphosphoramide mustard; PM, phosphoramide mustard; HPMA, 3-hydroxypropylmercapturic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; ALDH1, aldehyde dehydrogenase 1; GR, glutathione reductase.

Figure 5 depicts the constellation of interactions between BCNU and CY in the context of CY's own influence on its disposition when administered on successive days. CY induces the formation of HCY by inducing CYP3A and 2C (14,21). The decomposition product of HCY, acrolein, competitively inhibits ALDH1 thereby promoting the formation of additional acrolein. Direct inhibition of ALDH1 by BCNU further increases exposure to acrolein, which further promotes its own formation by inhibiting ALDH1. Since the depletion of glutathione by BCNU is dose dependent, a large single dose is more likely to deplete this nucleophile that otherwise would detoxify acrolein by forming a nontoxic conjugate.

The full complexity of the interaction between BCNU and CY described above has not been proved, but each individual potentially contributing effect depicted in Figure 5 has been. The surest way to avoid an interaction between these agents is to administer BCNU the day after the last dose of CY. However, preparative regimens of combinations of chemotherapeutic agents often are developed in the face of such interactions and their success or failure probably depends, to some extent, on the magnitude with which the interactions are expressed within a given individual. For example, the administration of low doses of BCNU over a 4-day course of CY administration is likely, based on our findings and calculations, to inhibit ALDH1, which may be important to the success of the regimen. Consideration of interactions of this sort while designing new clinical combinations of agents would allow more rational selection of doses, sequence of and interval between agents. Such interactions often can be anticipated from the known behavior of these agents and a few additional in vitro studies.

ACKNOWLEDGEMENT

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